described above. Lymphocytes, in which hTERT is normally strongly expressed (15), were used as a positive control.

Transferase-mediated deoxy uridine triphosphate nick end labelling

Apoptosis in HCC tissues was assessed using the transferase-mediated deoxy uridine triphosphate nick end-labelling technique (16). The signals were visualized with DAB and counterstained with haematoxylin. The apoptosis index was defined as the number of immunoreactive cells per 1000 scored cancer cells.

Telomere repeat amplification protocol assay

Telomerase activity was also semiquantitatively measured by a TRAP assay using a TRAPEZE Telomerase Detection Kit (Oncor, Gaithersburg, CA, USA) according to the manufacturer's protocol. Thirty frozen HCC samples were examined as described in supplementary method 1. Relative telomerase activities (RTAs) were classified into four groups: 0 (RTA was not detectable), 1+ ($0 < \text{RTA} < 50$), 2+ ($50 \leqq \text{RTA} < 100$) and 3+ ($100 \leqq \text{RTA}$).

Analysis of telomere length by quantitative fluorescence *in situ* hybridization

Telomeres were identified in the paraffin sections using a telomere-specific peptide nucleic acid fluorescence *in situ* hybridization kit as described previously (17). The sections were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) (1000 ng/ml, VYS-32-804830, ABBOTT JAPAN CO., Tokyo, Japan). Following the protocol of Meeker *et al.* (18), image-processed telomeric signals were quantified from digitized fluorescence microscopic images using the image analysis software package IP LABS (version 3.54, Scanalytics, Fairfax, VA, USA). Lymphocytes were used as internal controls of the telomeric signal because the telomere lengths of the lymphocytes were less affected by ageing than those of somatic cells (19). The telomeric pixel intensities of 15–20 nuclei of cancer cells and 5–10 nuclei of lymphocytes were recorded. To correct the different amounts of DNA in the sectioned nuclei, the telomeric signal intensity was modified by dividing the pixel intensity of the telomere signal for a given nucleus by the pixel intensity of the DAPI signal within that nucleus, as reported previously (18). Average telomere length was defined as Tel-T/Tel-L, where Tel-T and Tel-L are the average modified telomeric signal intensities of cancer cells and lymphocytes, respectively, measured in four different fields per sample.

Statistical analysis

The data are represented as the mean and SD in each category. The correlations of OS grade in HCC tissues to different clinicopathological and biochemical factors were assessed using the Kruskal–Wallis H test, the Spearman's Rank Correlation Coefficient and the Mann–Whitney U test. Differences of clinicopathological factors between hTERT-negative and hTERT-positive HCCs were analysed using the Mann–Whitney U test. $P < 0.05$ was considered to be statistically significant. All the tests were analysed using the STATVIEW program (Abacus Concepts, Berkeley, CA, USA).

Results

Immunohistochemical analysis of 8-hydroxy-2′-deoxyguanosine in human hepatocellular carcinoma samples

HCC tissues representative of each OS grade are shown in Figure 1a. 8-OHdG was mainly found in the nuclei of cancer cells, but it was also found in the nuclei of lymphocytes and fibroblasts and hepatocytes in non-cancerous tissues. The OS grades of the HCC tissues were: 15 Grade 0, 17 Grade 1, 15 Grade 2 and 21 Grade 3. None of several clinicopathological parameters of the 68 subjects (age, sex, etc.) was significantly related to the OS grade (Table 2). Furthermore, no tumour factors (differentiation of HCC tissues, size, stage or etiology) was significantly related to the OS grade (Table 3). On the other hand, the OS grade of non-cancerous tissues was significantly related to the patient's age, etiology and grading of chronic hepatitis ($P < 0.05$, Kruskal–Wallis H test and Spearman's rank correlation coefficient, Table 4).

Immunohistochemical analysis of human telomerase reverse transcriptase in human hepatocellular carcinoma samples

hTERT, an indicator of telomerase activity in cancer cells (14), was immunodetected in lymphocytes, in which it is normally expressed (15) (Fig. 1b, left panel), and in some cancer cells, but not in other cells, including normal hepatocytes. hTERT in HCC cells was mainly localized in the nucleus, especially in the nucleolus (Fig. 1b, right panel). It was also weakly detected in the cytoplasm of HCCs (Fig. 1b, left panel). Of the 68 HCC specimens examined, 47 were judged hTERT-positive (strong detection) and 21 were judged hTERT-negative (no or weak detection) (Table 3).

Oxidative stress shortens telomere length in human hepatocellular carcinoma samples

The telomere signals of cancer cells and lymphocytes were detected as green fluorescein isothiocyanate signals within the blue DAPI signals (the nuclear signals of cancer cells and lymphocytes) (Fig. 1c). Extensive OS was found to significantly accelerate the shortening of telomeres in cancer cells ($P < 0.001$, Kruskal–Wallis H test, Table 3). As the OS grade increased, the average telomere length of cancer cells strongly decreased in hTERT-negative HCCs ($P < 0.001$) whereas it did not

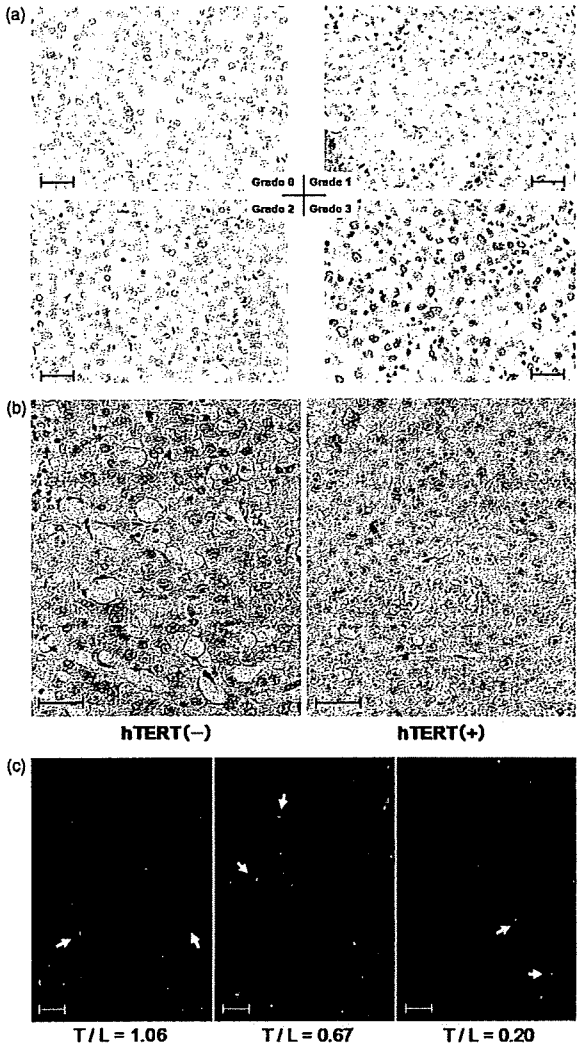


Fig. 1. Representative photographs of immunostaining and quantitative fluorescence *in situ* hybridization of human hepatocellular carcinoma (HCC) tissues. (a) Immunostaining of 8-hydroxy-2′-deoxyguanosine. The staining intensity was classified into four grades according to the percentage of immunoreactive cells. (b) Immunostaining of human telomerase reverse transcriptase (hTERT). In hTERT-negative HCCs, the cytoplasm of the cancer cell was faintly stained and the lymphocytes were strongly stained. In hTERT-positive HCCs, the nucleus, especially the nucleolus of the cancer cells, was strongly stained. (c) Representative photographs of quantitative fluorescence *in situ* hybridization for measurement of telomere length. White and red arrows in each photograph indicate the lymphocytes and the cancer cells in HCC tissues respectively. T/L below each photograph represents the average value of the mean Tel-T/mean Tel-L as the average telomere length of cancer cells in each HCC tissue, where mean Tel-T and mean Tel-L represent the average modified telomeric signal intensity of cancer cells and lymphocytes respectively. Scale bars, 50 μm.

significantly change in hTERT-positive HCCs (Fig. 2a). The difference between the average telomere lengths in hTERT-positive and hTERT-negative HCCs significantly increased with increasing OS grade ($P < 0.05$, Mann–Whitney U test, Fig. 2b).

Oxidative stress increases telomerase activity in human hepatocellular carcinoma samples

The immunodetection of hTERT in HCC tissues was significantly correlated with OS grade ($P < 0.001$, Kruskal–Wallis H test, Fig. 2b). Additionally, samples with higher OS grades had higher RTA scores in the TRAP assay ($P < 0.05$, Spearman's rank correlation coefficient, Fig. 2c). The degree of tumour differentiation was significantly related to hTERT expression in HCC tissues but was not related to the length of telomeres (Table 5).

Increased proliferative activity and apoptotic resistance in human hepatocellular carcinoma samples by oxidative stress through telomerase activation

We did not observe a correlation between the Ki-67 index, an indicator of tumoral proliferative activity, and OS grade in HCC tissues (Table 3), although we could not rule out the possibility that the Ki-67 index had a weak positive correlation with the OS grade in the hTERT-positive group and a weak negative correlation with the OS grade in the hTERT-negative group (Fig. 2d). On the other hand, OS was significantly correlated with the apoptosis index in HCC tissues ($P < 0.05$, Kruskal–Wallis H test, Table 3), especially in the hTERT-negative group ($P < 0.001$) (Fig. 2e). For OS Grades 2 and 3, the hTERT-positive HCC cells showed a significantly higher proliferative activity and a significantly higher apoptotic resistance than the hTERT-negative HCC cells ($P < 0.05$, Mann–Whitney U test, Fig. 2f). Additionally, malignancy parameters of HCC tissues (Ki-67 index, apoptosis index, differentiation and stage) were not significantly different between the OS Grades 0–1 and 2–3 groups in HCC tissues (Table 6). However, the Ki-67 index and differentiation were significantly different between the OS Grade 0–1 group and the OS Grade 2–3 and hTERT-positive group in HCC tissues ($P < 0.05$, Mann–Whitney U test, Table 6).

Downexpression of phosphatase and tensin homolog deleted on chromosome 10 and activated phosphorylation of AKT in human hepatocellular carcinoma samples by oxidative stress

Phospho-AKT (P-AKT), a regulator of telomerase activity (20, 21), was detected in the nucleus and the cytoplasm of cancer cells (Fig. 3a, panel A). PTEN, an inhibitor of Akt phosphorylation (22), was not detected in the same lesions (panel B). On the other hand, PTEN was detected strongly in the cytoplasm and weakly in the nucleus in cancer cells that were P-AKT negative (panels C and D). Staining of PTEN was stronger in the non-cancerous part of an HCC sample (N in panel E) than in the cancerous part (T), as reported previously (23). As

Table 2. The correlation between the oxidative stress grades of 68 human hepatocellular carcinoma samples and the clinicopathological parameters of their human hepatocellular carcinoma patients

	8-OHdG				
	Grade 0 ($n=15$)	Grade 1 ($n=17$)	Grade 2 ($n=15$)	Grade 3 ($n=21$)	*P*-value
Sex (M/F)	11/4	11/7	10/6	11/10	0.5808
Age (years)	61.9 ± 8.4	64.1 ± 6.3	59.3 ± 13.0	63.6 ± 6.3	0.6465
BMI (kg/m^2)	22.9 ± 3.6	23.7 ± 2.7	21.9 ± 3.2	24.0 ± 2.3	0.3415
AST (IU/L)	63.4 ± 35.1	62.9 ± 43.5	62.5 ± 42.9	45.5 ± 22.7	0.5273
ALT (IU/L)	70.3 ± 39.8	53.6 ± 29.0	58.3 ± 42.1	40.8 ± 23.0	0.1700
PLT (× 10^4/μl)	13.6 ± 6.3	12.8 ± 4.8	13.4 ± 6.3	12.9 ± 7.1	0.9507
ALB (g/dl)	3.85 ± 0.49	4.20 ± 0.47	4.15 ± 0.28	4.15 ± 0.43	0.2689
ICG15 (%)	22.8 ± 13.5	13.9 ± 8.1	17.5 ± 12.1	14.6 ± 12.1	0.2684
AFP (μg/ml)	5860 ± 19 708	7458 ± 2215	294 ± 897	11 936 ± 46 136	0.7615
PIVKA-II (mAU/ml)	1211 ± 4372	1936 ± 5316	334 ± 510	394 ± 1113	0.9807

Data are expressed as mean ± SD.
Statistical analysis was performed using the Kruskal–Wallis *H*-test.
ALB, albumin; ALT, alanine aminotransferase; AFP, á-foetoprotein ; AST, aspartate aminotransferase; BMI, body mass index; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; F, female; ICG, indocyanine green; M, male; PLT, platelet; SD, standard deviation.

Table 3. Correlations between the oxidative stress grades of 68 human hepatocellular carcinoma samples and clinicopathological factors

	8-OHdG				
	Grade 0 ($n=15$)	Grade 1 ($n=17$)	Grade 2 ($n=15$)	Grade 3 ($n=21$)	*P*-value
T-length (T/L)	1.097 ± 0.198	0.599 ± 0.271	0.549 ± 0.209	0.822 ± 0.663	0.00008110**
hTERT (N/P)	15/0	15/2	10/5	7/14	0.00006758**
Telomerase (0/1/2/3)	2/3/0/0	1/4/1/0	2/2/3/1	0/4/5/2	0.002539*
Ki-67 index	13.41 ± 8.43	13.41 ± 7.14	14.74 ± 9.87	15.75 ± 9.09	0.8654
Apo index	1.218 ± 0.720	1.782 ± 0.913	2.526 ± 1.372	2.306 ± 1.976	0.04040*
Different (W/M/P)	9/5/1	7/8/2	7/5/3	6/10/5	0.05515
Size (mm)	44.53 ± 43.00	49.47 ± 37.81	45.86 ± 36.17	41.95 ± 20.12	0.5474
Stage (I/II/III/IV)	5/7/3/0	4/5/7/1	1/11/3/0	3/14/2/2	0.6141
Aetiology (B/C/B+C/NBNC)	3/9/1/2	2/13/0/2	5/9/0/1	4/14/0/3	0.5311
Non-tumour (N/CH/LC)	1/5/9	0/9/8	1/5/9	0/12/9	0.2674

Data are expressed as mean ± SD.
Statistical analysis was performed using the Spearman's rank correlation coefficient in telomerase, differentiation, stage and non-tumour and using the Kruskal–Wallis *H*-test in other factors.
*$P < 0.05$.
**$P < 0.001$.
Apo index, apoptosis index; B/C/B+C/NBNC, HBV/HCV/HBV+HCV/non-HBV, non-HCV; Different, tumour differentiation; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; HBV, hepatitis B virus; HCV, hepatitis C virus; hTERT, human telomerase reverse transcriptase; N/P, negative/positive; NL/CH/LC, normal liver/chronic hepatitis/liver cirrhosis; SD, standard deviation; T-length, telomere length; T/L, tumour cell/lymphocyte; W/M/P, well differentiated/moderately differentiated/poorly differentiated.

the OS grade increased, the level of Akt phosphorylation in HCC samples increased significantly ($P < 0.001$, Fig. 3b) and the expression of PTEN in HCC samples decreased significantly ($P < 0.05$, Fig. 3b). These results suggest that the activation of telomerase by OS in HCC tissues is due to the downexpression of PTEN and the following activation of Akt.

Discussion

Although OS has been associated with chronic liver disease (24, 25), whether it has a role in the clinicopathology of HCC has not been investigated previously. In the present study, most HCC tissues (53/68, 77.9%) had high levels of OS (OS grades over 1). However, the 8-OHdG levels in the cancerous parts were not significantly related to those in the non-cancerous parts (Table 4). This suggests that the cause of OS in HCC cells is different from that in hepatocytes in chronic liver diseases.

Furthermore, the OS grade in HCC tissues was not significantly correlated with several clinicopathological factors of HCC samples, such as degree of differentiation, size, stage, etiology and type of non-cancerous tissue

Table 4. The correlation between the oxidative stress grades of 68 non-cancerous tissues and the clinicopathological parameters of their patients

	8-OHdG				
	Grade 0 ($n = 14$)	Grade 1 ($n = 22$)	Grade 2 ($n = 19$)	Grade 3 ($n = 13$)	*P*-value
Sex (M/F)	9/5	13/9	10/9	10/3	0.6944
Age (years)	60.0 ± 6.4	60.2 ± 9.4	66.1 ± 7.0	64.2 ± 7.2	0.04901*
BMI (kg/m^2)	23.5 ± 2.1	21.9 ± 2.8	23.9 ± 2.7	24.1 ± 2.7	0.1493
AST (IU/L)	61.0 ± 46.0	58.2 ± 36.3	57.1 ± 36.3	66.0 ± 33.6	0.7817
ALT (IU/L)	60.5 ± 38.5	53.0 ± 29.8	54.6 ± 40.1	65.4 ± 37.8	0.7232
PLT (× 10^4/μl)	13.9 ± 8.5	12.4 ± 5.8	14.8 ± 4.9	12.0 ± 5.4	0.2568
ALB (g/dl)	4.12 ± 0.28	4.11 ± 0.55	4.05 ± 0.45	4.18 ± 0.32	0.7337
ICG15 (%)	22.8 ± 13.5	13.9 ± 8.1	17.5 ± 12.1	14.6 ± 12.1	0.2684
Aetiology (B/C/B+C/NBNC)	5/9/0/0	8/12/2/0	1/14/3/1	0/11/2/0	0.01378*
Grading (A0/A1/A2/A3)	0/11/2/1	0/12/9/1	1/8/9/1	1/2/5/5	0.003754*
Staging (F0/F1/F2/F3/F4)	0/2/4/0/8	0/2/4/6/10	1/1/5/3/9	1/0/0/3/9	0.1893
OS tumour (G0/G1/G2/G3)	4/2/3/5	6/8/2/6	4/5/4/6	1/2/6/4	0.1679

Data are expressed as mean ± SD.

Statistical analysis was performed using the Spearman's rank correlation coefficient in grading, staging and OS tumour and using the Kruskal–Wallis *H*-test in other factors.

*$P < 0.05$.

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; B/C/B+C/NBNC, HBV/HCV/HBV+HCV/non-HBV, non-HCV; BMI, body mass index; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; HBV, hepatitis B virus; HCC, human hepatocellular carcinoma; HCV, hepatitis C virus; ICG, indocyanine green; OS, oxidative stress; OS tumour, 8-OHdG grade of HCC tissue; PLT, platelet; SD, standard deviation.

(Table 3), although the OS grade in non-cancerous tissues was significantly correlated with several clinicopathological factors (Table 4). Tumour cells produce ROS at a far greater rate than do non-tumour cells *in vitro* and *in vivo* (26, 27). The increased ROS in HCC tissues may be due to hypoxia (28), which arises from the high proliferative activity and high cellular density of cancer cells. OS is also a mediator of angiogenesis signalling (29), which suggests that one of the effects of OS is to counteract the effects of hypoxia.

Our findings that OS in HCC tissues significantly accelerated telomere shortening and increased the apoptosis of cancer cells (Table 3) are consistent with our previous reports that OS hastened the telomere shortening of hepatocytes in chronic hepatitis C and non-alcoholic fatty liver disease (30, 31). Thus, OS appears to promote the senescence of cancer cells in HCC tissues through telomere shortening, as it does in hepatocytes in chronic liver diseases. OS also inhibits the proliferation of normal hepatocytes through telomere shortening (30). However, OS was not associated with the proliferative activity of HCC cells in this study, including hTERT-negative HCCs (Fig. 2d). Cancer cells with a high proliferative activity appear to be in a hypermetabolic state and appear to generate much cellular ROS. Additionally, the cell cycle checkpoint usually breaks down during carcinogenesis. Therefore, HCCs with high levels of OS may proliferate despite the OS-induced telomere shortening.

As the OS grade increased, the immunodetection of hTERT and telomerase activity in HCC tissues increased (Table 3). Although hTERT mRNAs were detected in the hepatocytes and in the serum of patients with chronic liver disease (32, 33), we were unable to detect hTERT protein in non-cancerous parts including liver tissues from patients with chronic hepatitis or liver cirrhosis. These results suggest that hTERT is immunodetected much more strongly in HCC tissues than in non-cancerous tissues. In agreement with previous reports (34, 35), HCC cells in the hTERT-positive group tended to have a higher proliferative activity and higher apoptotic resistance than did those in the hTERT-negative group, particularly in a highly oxidative-stressed environment (Fig. 2f). Additionally, HCC tissues with a high OS grade (2–3) and a high immunodetection of hTERT were significantly more proliferative and dedifferentiated than those with a low OS grade (0–1) (Table 6). Thus, OS in HCC tissues may increase the malignant potential of cancer cells by upregulating hTERT.

Although there is evidence that OS contributes to telomerase activation in HCC (36), the mechanism is unknown. In this study, we regarded the immunodetection of hTERT in the nucleus of cancer cells as an indicator of activated telomerase because the nuclear localization of hTERT is required to promote the elongation of telomere sequences within the cellular nucleus. hTERT must be phosphorylated before it can be translocated from the cytoplasm to the nucleus (37). Protein kinase B/Akt is known to be involved in the phosphorylation and the expression of hTERT (20, 21). Our finding that Akt is activated in HCC tissues with high-grade OS (Fig. 3b) is consistent with a report that OS increased the phosphorylation of Akt in human hepatoma cells *in vitro* (38). Additionally, the expression

of PTEN, a tumour suppressor and an inhibitor of the phosphorylation of Akt (22), significantly decreased in HCC tissues with increasing OS grade (Fig. 3b). OS may induce the inactivation of PTEN and the following activation of AKT in cancer cells (39). The downregulation and functional inactivation of PTEN may be a major mechanism of Akt phosphorylation by OS. Therefore, we assume that the PTEN–Akt pathway is a key factor in the activation of telomerase by OS in HCC tissues.

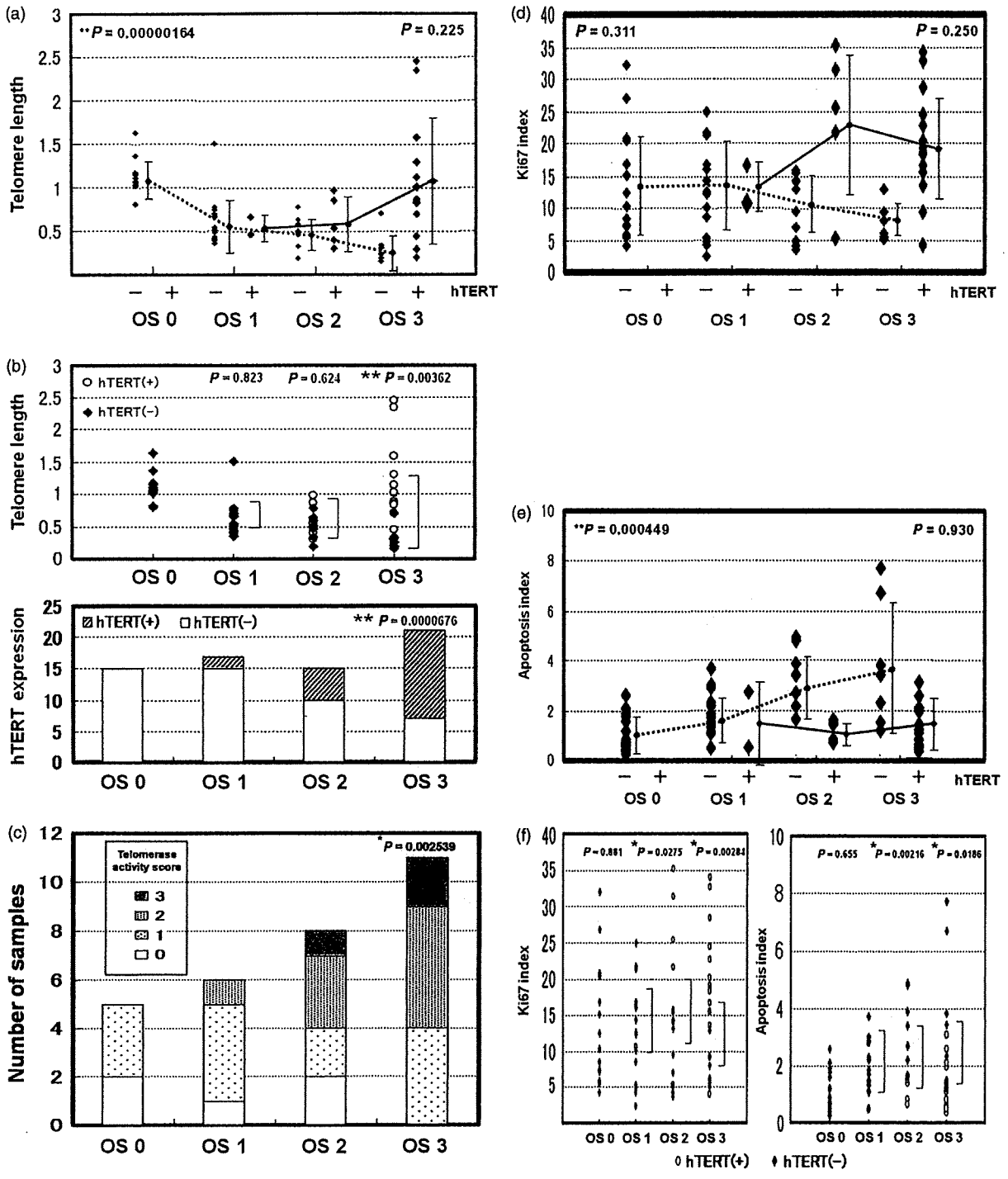


Table 5. Human telomerase reverse transcriptase expression but not telomere length is related to the degree of tumour differentiation in 68 human hepatocellular carcinoma samples

	Histological grade of carcinoma			
Measurement	Well differentiated ($n = 15$)	Moderately differentiated ($n = 17$)	Poorly differentiated ($n = 15$)	P-value†
Telomere length‡	0.713 ± 0.329	0.835 ± 0.490	0.734 ± 0.653	0.4612
hTERT (N/P)§	24/5	18/10	5/6	0.01847*

†Statistical analysis was performed using the Kruskal–Wallis H-test.
‡Defined as T/L, where T and L are the telomere signal intensities of tumour cells and lymphocytes respectively. Data are expressed as mean ± SD.
§N/P, expression of hTERT in HCC tissues is negative/positive.
*$P < 0.05$.
HCC, human hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; SD, standard deviation.

Table 6. Correlations among the oxidative stress grade of human hepatocellular carcinomas, expression of human telomerase reverse transcriptase and clinicopathological factors

		Grades 2–3			
Measurement	Grades 0–1 ($n = 32$)	hTERT (−) ($n = 13$)	P-value	hTERT (+) ($n = 19$)	P-value
Ki-67 index	13.41 ± 7.65	15.33 ± 9.30	0.5349	20.57 ± 9.30	0.009035*
Apo index	3.591 ± 6.712	2.389 ± 1.695	0.2031	1.425 ± 0.886	0.3258
Differentiation (W/M/P)	16/13/1	13/15/8	0.05286	3/9/2	0.03283*
Stage (I/II/III/IV)	9/12/10/1	4/25/3/2	0.8865	0/14/3/2	0.3194

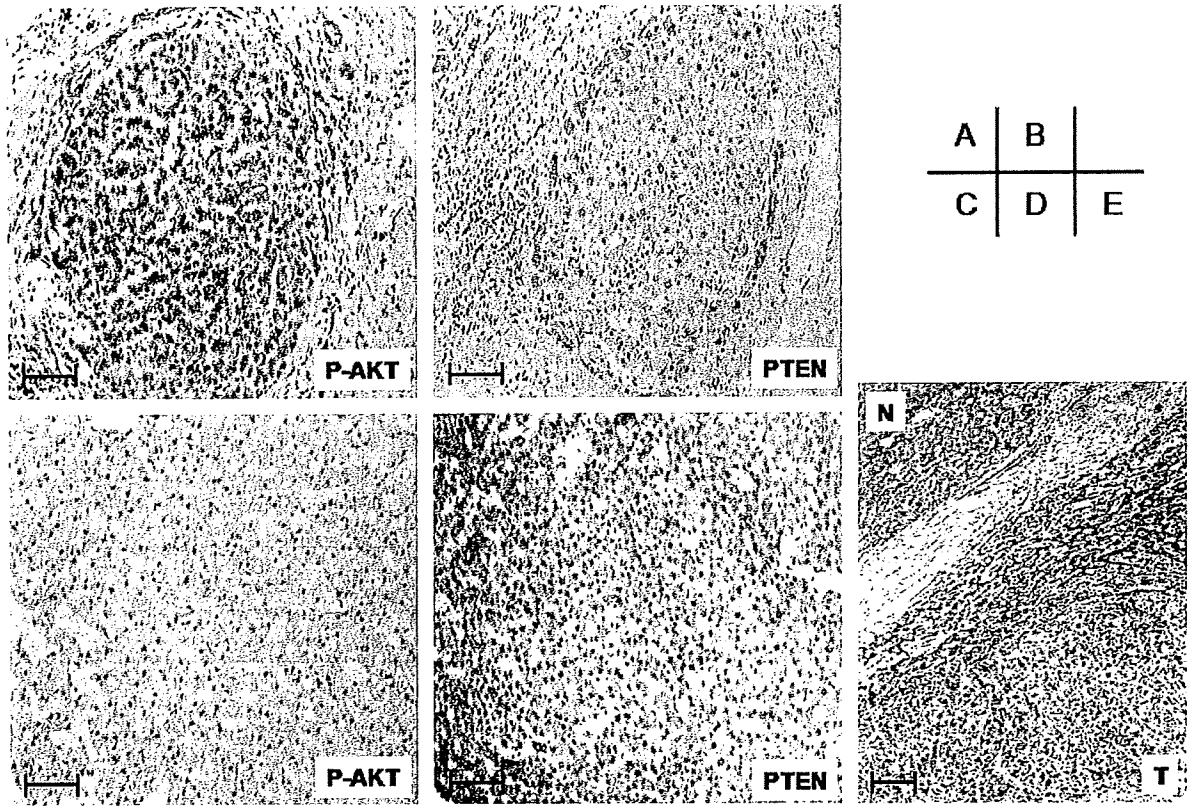
Data are expressed as mean ± SD.
Statistical analysis was performed using the Mann–Whitney U-test.
*$P < 0.05$.
Apo index, apoptosis index; differentiation, tumour differentiation; hTERT, human telomerase reverse transcriptase; W/M/P, well differentiated/moderately differentiated/poorly differentiated.

Our findings suggest that OS is associated with the malignant potential of HCCs through the activation of telomerase. Several drugs that are antioxidants are reported to prevent hepatocarcinogenesis and inhibit the growth of HCC cells (40, 41). We also recently demonstrated that epigallocatechin-3-gallate, a major polyphenol of green tea and a potent antioxidant, suppressed the growth of HCC cells *in vitro* and *in vivo* (42). These results raise the possibility of using antioxidant therapy to reduce OS in HCCs with high telomerase activity in order to inhibit the growth of HCCs. This is supported by the findings that the anticancer effects of antioxidants partially result from the downregulation of telomerase activity (43) and that inhibition of telomerase enhances the effects of chemotherapeutic agents in various cancer cells (44).

In conclusion, our findings show that HCC tissues are frequently characterized by OS, which contributes to the acceleration of telomere shortening and the activation of telomerase in cancer cells. The activation of telomerase by OS possibly results from the downexpression of PTEN and the phosphorylation of Akt. Because HCC cells that have high levels of hTERT as a result of OS are more malignant, continuous OS during the progression of HCCs may indicate a poor prognosis. Our results suggest that OS in HCC tissues can be used as a measure of the malignant potential.

Fig. 2. Oxidative stress (OS) accelerated telomere shortening, increased telomerase activity in human hepatocellular carcinoma (HCC) cells and was related to the increased proliferative activity and apoptotic resistance in HCC tissues through telomerase activation. (a) In human telomerase reverse transcriptase (hTERT)-negative HCCs, the average telomere length significantly decreased with increasing OS grade (dotted line) and in hTERT-positive HCCs, it elongated (solid line) (*$P < 0.05$, **$P < 0.001$ by the Kruskal–Wallis H test). (b) The average telomere length in hTERT-positive HCCs was elongated compared with those in hTERT-negative HCCs as the OS grade increased (upper) (by the Mann–Whitney U test). There was a positive correlation statistically between the OS grade and the expression of hTERT in HCC tissues (lower) (**$P < 0.001$ by the Kruskal–Wallis H test). (c) Distribution of telomerase activity among 30 HCC samples. Higher telomerase activity scores were more frequently observed in samples with higher OS grades (*$P < 0.05$ by Spearman's rank correlation coefficient). (d) The Ki-67 index in hTERT-negative HCCs (dotted line) and hTERT-positive HCCs (solid line) were not significantly affected by the OS grade (Kruskal–Wallis H test). (e) As the OS grade increased, the apoptosis index increased significantly in hTERT-negative HCCs (dotted line) but did not significantly change in hTERT-positive HCCs (solid line) (*$P < 0.05$, **$P < 0.001$ by the Kruskal–Wallis H test). (f) The Ki-67 index increased and the apoptosis index decreased in hTERT-positive HCCs compared with those in hTERT-negative HCCs, as the OS grade increased (*$P < 0.05$ by the Mann–Whitney U test). The data in (a), (d) and (e) show the mean and SD.

(a)



(b)

OS grade	P-AKT		PTEN	
	(–)	(+)	(–)	(+)
0 (*n*=15)	14	1	3	12
1 (*n*=17)	13	4	4	13
2 (*n*=15)	10	5	8	7
3 (*n*=21)	6	15	15	6
P-value	0.00013**		0.00435*	

Fig. 3. Oxidative stress (OS) increased the level of Akt phosphorylation and decreased the expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in human hepatocellular carcinoma (HCC) samples. (a) Representative photographs of immunostainings of phospho-Akt (P-AKT) in Ser473 and PTEN. P-AKT was detected in the nucleus and in the cytoplasm of cancer cells in the P-AKT-positive sample (A) and PTEN was not detected in the same lesion (B). Panel C shows a P-AKT-negative sample and PTEN was detected mainly in the cytoplasm of the cancer cells in the same lesion (D). Staining of PTEN was stronger in the non-cancerous part (N) than in the cancerous part (T) of the HCC sample (E). Scale bars, 50 μm. (b) The phosphorylation of Akt in HCC tissues significantly increased and the expression of PTEN in HCC tissues significantly decreased as the OS grade increased (*$P < 0.05$, **$P < 0.001$ by the Kruskal–Wallis H test).

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Hepatol Int (2009) 3:445–452
DOI 10.1007/s12072-009-9135-0

Antiviral activity, dose–response relationship, and safety of entecavir following 24-week oral dosing in nucleoside-naive Japanese adult patients with chronic hepatitis B: a randomized, double-blind, phase II clinical trial

Michiko Shindo · Kazuaki Chayama · Satoshi Mochida · Joji Toyota · Eiichi Tomita · Hiromitsu Kumada · Osamu Yokosuka · Michio Sata · Norio Hayashi · Kazuyuki Suzuki · Takeshi Okanoue · Hirohito Tsubouchi · Hiroki Ishikawa · Taku Seriu · Masao Omata

Received: 21 January 2009 / Accepted: 7 May 2009 / Published online: 23 May 2009

Abstract
Purpose A randomized, double-blind, multicenter study (ETV-047) was conducted to evaluate the dose–response relationship of entecavir and compare its antiviral activity and safety with lamivudine in Japanese patients with chronic hepatitis B (CHB).
Methods One hundred thirty-seven nucleoside-naive adult patients with CHB were randomized to once-daily oral doses of entecavir 0.01, 0.1, or 0.5 mg or lamivudine 100 mg for 24 weeks. The primary efficacy end point used to evaluate the dose–response relationship was mean change from baseline in serum hepatitis B virus (HBV) DNA level at week 22, as determined by polymerase chain reaction assay.
Results Entecavir demonstrated a clear dose–response relationship, with mean change from baseline in serum

M. Shindo (✉)
Division of Liver Disease, Department of Internal Medicine, Akashi Municipal Hospital, 1-33 Takasyo-machi, Akashi-shi, Hyogo, Japan
e-mail: mshindo@skyblue.ocn.ne.jp

K. Chayama
Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

S. Mochida
Department of Gastroenterology and Hepatology, Saitama Medical University, Saitama, Japan

J. Toyota
Department of Hepatology, Sapporo Kosei General Hospital, Hokkaido, Japan

E. Tomita
Department of Gastroenterology, Gifu Municipal Hospital, Gifu, Japan

H. Kumada
Department of Hepatology, Toranomon Hospital, Tokyo, Japan

O. Yokosuka
Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan

M. Sata
Department of Gastroenterology, Kurume University School of Medicine, Fukuoka, Japan

N. Hayashi
Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Osaka, Japan

K. Suzuki
Department of Internal Medicine, Iwate Medical University, Iwate, Japan

T. Okanoue
Department of Gastroenterology, Saiseikai Suita Hospital, Osaka, Japan

H. Tsubouchi
Department of Digestive Disease and Lifestyle-Related Disease, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

H. Ishikawa · T. Seriu
Pharmaceutical Research Institute, Bristol-Myers Squibb Japan, Tokyo, Japan

M. Omata
Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

HBV DNA level of −3.11, −4.77, and −5.16 $\log_{10}$ copies/ml with entecavir 0.01, 0.1, and 0.5 mg, respectively. Entecavir 0.5 mg was superior to lamivudine 100 mg for the mean change in HBV DNA level (−5.16 vs. −4.29 $\log_{10}$ copies/ml; $P = 0.007$). The overall incidence of adverse events was comparable between treatment groups. Two patients discontinued treatment because of adverse events (one with liver cirrhosis [entecavir 0.5 mg] and one with grade 4 serum alanine aminotransferase (ALT) elevation, nausea, and malaise [lamivudine 100 mg]). Serum ALT flares were observed in four patients; flares were associated with 2 $\log_{10}$ reductions or more in HBV DNA level and resolved without dose interruption.

Conclusion Entecavir 0.01–0.5 mg is well tolerated and produces a dose-dependent reduction in viral load in nucleoside-naive Japanese patients with CHB. Compared with lamivudine 100 mg, entecavir 0.1 mg demonstrated noninferiority and entecavir 0.5 mg was superior in this population.

Keywords Chronic hepatitis B · Entecavir · Lamivudine · HBV DNA · ALT flare

Introduction

It is reported that more than 2 billion individuals worldwide have been infected with hepatitis B virus (HBV) and approximately 350 million people are long-term HBV carriers [1]. Chronic hepatitis B (CHB) is induced by chronic replication of HBV in the liver and has a poor prognosis, with 20–40% of infected individuals developing liver cirrhosis, noncompensated liver disorder, or hepatocellular carcinoma [2]. Treatment of CHB is aimed at sustained inhibition of HBV replication and remission of liver disease [3], ultimately preventing progression to liver cirrhosis or hepatocellular carcinoma [4].

Prior to the advent of the nucleoside analog lamivudine, interferon-α formed the mainstay of treatment, but this immunoregulatory cytokine requires parenteral administration and is poorly tolerated [5]. Lamivudine is well tolerated on oral administration and has been proven to be highly effective in the treatment of CHB, but the emergence of resistance mutations (including the YMDD motif) in the reverse-transcriptase domain of HBV polymerase frequently results in overt viral rebound and disease progression [6–9]. The novel nucleoside analog adefovir is effective against wild-type HBV and lamivudine-resistant strains and is well tolerated on long-term administration, but its clinical use is restricted by the need for renal monitoring in patients with impaired renal function [10].

Entecavir, a cyclopentylguanine-derived nucleoside analog and selective inhibitor of HBV replication, was approved by the U.S. Food and Drug Administration in 2005 for the treatment of CHB. Entecavir displays potent antiviral activity in the woodchuck and duck models of HBV infection [11, 12] and is reported to be 100- to 2,200-fold more potent than lamivudine and adefovir in inhibiting HBV replication in vitro [13, 14]. Phase II clinical trials of entecavir conducted in non-Japanese patients with CHB have demonstrated entecavir to be well tolerated and more effective than lamivudine [15, 16].

A global dose-finding study (ETV-005) conducted in lamivudine-naive patients with CHB compared three doses of entecavir (0.01, 0.1, and 0.5 mg once daily) with lamivudine 100 mg once daily over a 22-week treatment period. Entecavir showed a clear dose–response relationship and was well tolerated at all three dose levels; in addition, 0.1 and 0.5 mg of entecavir showed superior antiviral activity compared with 100 mg of lamivudine [15].

Phase I studies of single-dose (0.05–2.5 mg) and multiple-dose (0.1–1.0 mg daily) entecavir conducted in Japan have confirmed the drug's safety in healthy men. As in Caucasian populations, entecavir displayed linear plasma pharmacokinetics over a wide range of doses, including putative therapeutic doses (0.5 and 1.0 mg), in Japanese subjects; there was no evidence of significant ethnic differences in its pharmacokinetics and pharmacodynamics. Similar findings to those obtained in the global phase II clinical trials of entecavir might therefore be expected from corresponding studies conducted in Japanese patients.

To evaluate the dose–response relationship, the antiviral activity and safety of entecavir in Japanese CHB patients, we conducted a 24-week phase II study comparing entecavir (0.01, 0.1, and 0.5 mg daily) to lamivudine (100 mg daily).

Materials and methods

Study design

This randomized, double-blind, double-dummy study was conducted at 38 institutions in Japan from August 2003 to March 2005. Eligible patients comprised 20- to 75-year-old men and women with CHB who fulfilled the following criteria: (i) HBsAg-positive for 24 weeks or more or IgM HBcAb-negative with biopsy-confirmed CHB; (ii) HBeAg-positive or HBeAg-negative for 12 weeks or more; (iii) serum HBV DNA level 40 MEq/ml or more (143 pg/ml) by Quantiplex™ branched DNA hybridization method (bDNA assay) ($\geq$7.6 $\log_{10}$ genome equivalent by the transcription-mediated amplification method or $\geq 10^{7.6}$ copies/ml by Roche Amplicor™ polymerase chain reaction method [PCR assay]) measured 2 weeks or more before screening and serum HBV DNA level 40 MEq/ml or more (by bDNA assay) at screening; (iv) serum alanine

aminotransferase (ALT) level 1.25–10 times the upper limit of normal (ULN); and (v) well-compensated liver disease with prothrombin time prolongation 3 s or less or international normalized ratio 1.5 or less, serum albumin level 3.0 g/dl or more, and total bilirubin 2.5 mg/dl or less (42.75 μmol/l). After a 6-week screening period, eligible patients were stratified according to HBeAg status and study site and randomized (1:1:1:1) to oral treatment with entecavir (0.01, 0.1, or 0.5 mg plus matching placebo capsule) or lamivudine (100 mg plus matching placebo tablet) once daily for 24 weeks. All doses were administered at fixed times of the day, avoiding the 2 h before and after meals. Pregnant women were excluded from the study, as were patients with liver cirrhosis, patients with a history or evidence of variceal bleeding, patients with hepatic encephalopathy or ascites requiring diuretics, or patients with paracentesis. Patients with other liver disease (e.g., autoimmune hepatitis) were excluded from the study. In addition, patients were excluded if they had a serum creatinine level more than 1.5 × ULN, hemoglobin level less than 10.0 g/dl, platelet count less than 70,000/mm^3, granulocyte count less than <1,500/mm^3 or plasma α-fetoprotein level more than 100 ng/ml, a history of allergy induced by nucleoside analog or exposure to nucleoside analogs, a recent history (previous 24 weeks) of treatment with immunosuppressives or interferon-α/β, or current treatment of CHB.

Treatment efficacy was assessed after 22 weeks, and all eligible patients who completed 24 weeks of blinded therapy were given the option of enrolling in a separate entecavir trial. Patients who discontinued therapy prematurely were followed up for 24 weeks postdosing. Patients began anti-HBV therapy as recommended by their physician during the postdosing follow-up period.

Informed consent was obtained from all patients in writing prior to their inclusion in the study. The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines and notifications were issued by the Ministry of Health and Labor.

Efficacy and safety assessment

The primary efficacy end point for the evaluation of the dose–response relationship of entecavir was the change from baseline in mean serum HBV DNA level at week 22, as determined by PCR assay. Secondary efficacy end points for the assessment of the noninferiority of entecavir at each dose to lamivudine included the change from baseline in mean serum HBV DNA level at week 22, as determined by PCR assay, the percentage of patients with a reduction in serum HBV DNA level 2 $\log_{10}$ copies/ml or more or a serum HBV DNA level below the limit of detection (400 copies/ml by PCR assay; 2.5 pg/ml or 0.7 MEq/ml by bDNA assay) at week 22, the percentage of patients with HBeAg loss, the percentage of patients with HBeAg seroconversion (HBeAg loss and appearance of HBe-antibody), the percentage of patients achieving ALT normalization (World Health Organization grade 0: <1.25 × ULN), and the percentage of patients achieving a protocol-defined response (HBV DNA level <0.7 MEq/ml by bDNA assay, HBeAg negativity and serum ALT level <1.25 × ULN for HBeAg-positive patients; HBV DNA level <0.7 MEq/ml by bDNA assay and serum ALT level <1.25 ULN for HBeAg-negative patients) at week 22. The incidence of genotypic drug resistance was also assessed in patients who had a 1 $\log_{10}$ copies/ml or more increase in HBV DNA by PCR from nadir while on study drug.

Based on the results of the global dose–response study of entecavir conducted in nucleoside-naive patients (ETV-005 study) [15], noninferiority of entecavir 0.1 or 0.5 mg compared with lamivudine (100 mg) was confirmed if the upper 95% confidence interval (CI) for the difference in mean HBV DNA levels at week 22 was 0.8 $\log_{10}$ copies/ml or less.

Assay methods

Serum HBV DNA level was determined by Roche Amplicor™ PCR assay (Roche Diagnostics K.K., Tokyo, Japan) and Quantiplex™ (Chiron) bDNA assay. Clinical laboratory tests, serum HBV DNA assays, and HBV serology were performed at the central clinical laboratory designated by the trial sponsor. Genotypic analysis of HBV isolates was performed using samples collected from patients on the first day of treatment. Genotypic analysis of HBV DNA polymerase was performed at SRL Inc. (Tokyo, Japan).

Statistical analysis

Numerical data were expressed by descriptive statistics. Serum HBV DNA level, a continuous variable, was analyzed after logarithmic transformation. For treatment group, comparisons of continuous variables, analysis of variance models, incorporating baseline HBV DNA level and HBeAg status as covariates were employed. For intertreatment comparisons of binary data, Cochran–Mantel–Haenszel tests were employed using baseline HBeAg status as a stratification factor. For analysis of dose–response relationships, Student's t test was applied to linear regression plots of serum HBV DNA level against log dose. A two-sided $P < 0.05$ was taken to indicate statistical significance. For analysis of dose–response relationships using efficacy data, a two-sided $P < 0.05/3$ was taken to

indicate statistical significance following Bonferroni adjustment.

Results

Study population and demographic characteristics

A total of 137 patients, including 20- to 73-year-old men and women, met the study eligibility criteria and were randomized to the following treatment groups: entecavir 0.01 mg ($n = 35$), entecavir 0.1 mg ($n = 34$), entecavir 0.5 mg ($n = 34$), and lamivudine 100 mg ($n = 34$). Three patients (two in the entecavir 0.5 mg group and one in the lamivudine 100 mg group) discontinued the study prematurely; the reasons for discontinuation were noncompliance (one patient in the entecavir 0.5 mg group) and adverse events (liver cirrhosis in one patient [entecavir 0.5 mg group] and grade 4 serum ALT elevation with nausea and malaise in one patient [lamivudine 100 mg group]). Accordingly, a total of 134 patients (entecavir 0.01 mg group, 35 patients; entecavir 0.1 mg group, 34 patients; entecavir 0.5 mg group, 32 patients; and lamivudine 100 mg group, 33 patients) completed 24 weeks of treatment and were included in the efficacy assessment.

The four treatment groups were matched with respect to gender, age, body weight, and proportion of HBeAg-positive patients (Table 1). Serum HBV DNA levels by PCR assay (mean ± SD) at baseline were 7.94 ± 0.87, 8.09 ± 1.05, 8.39 ± 0.73, and 7.94 ± 0.83 $\log_{10}$ copies/ml for the entecavir 0.01, 0.1, and 0.5 mg and lamivudine 100 mg groups, respectively. With regard to HBV genotype, 124 patients were genotype C, 6 patients were genotype A, 5 patients were genotype B, and 2 patients were genotype F. All patients were nucleos(t)ide-naive and none had been pretreated with interferon therapy.

Virologic response

Mean changes (from baseline) in serum HBV DNA level at week 22 were −3.11, −4.77, and −5.16 $\log_{10}$ copies/ml with entecavir 0.01, 0.1, and 0.5 mg, respectively (Fig 1; Table 2). Estimated differences in serum HBV DNA levels between the 0.1 and 0.5 mg entecavir groups and the low-dose entecavir group (0.01 mg) were determined after adjustment for baseline level and HBeAg status. Estimated intertreatment group differences (adjusted 95% CI) were −1.61 (−2.20 to −1.02) $\log_{10}$ copies/ml between the entecavir 0.01 and 0.1 mg groups and −1.95 (−2.53 to −1.37) $\log_{10}$ copies/ml between the entecavir 0.5 and 0.01 mg groups; both of these differences were statistically significant ($P < 0.0001$). In contrast, the difference in serum HBV DNA levels between the high-dose (0.5 mg) and medium-dose (0.1 mg) entecavir groups was not statistically significant (estimated difference [adjusted 95% CI] −0.23 [−0.69 to 0.23] $\log_{10}$ copies/ml). Taken together, these results demonstrate the superiority of high- and medium-dose entecavir (0.1 and 0.5 mg) compared with low-dose entecavir (0.01 mg) in terms of viral load reduction (Table 3). Linear regression analyses indicated a

Table 1 Baseline demographics and clinical characteristics of treated subjects

	ETV 0.01 mg ($n = 35$)	ETV 0.1 mg ($n = 34$)	ETV 0.5 mg ($n = 34$)	LVD 100 mg ($n = 34$)
Male, *n* (%)	25 (71.4)	23 (67.6)	23 (67.6)	28 (82.4)
Female, *n* (%)	10 (28.6)	11 (32.4)	11 (32.4)	6 (17.6)
Age (years), mean ± SD	42.0 ± 12.5	40.1 ± 9.8	39.8 ± 10.4	42.3 ± 12.6
Weight (kg), mean ± SD	66.2 ± 12.5	64.6 ± 11.9	65.3 ± 11.1	64.4 ± 9.0
Ethnicity Japanese, *n* (%)	35 (100)	34 (100)	34 (100)	34 (100)
HBV DNA ($\log_{10}$ copies/ml by PCR), mean ± SD	7.94 ± 0.87	8.09 ± 1.05	8.39 ± 0.73	7.94 ± 0.83
HBeAg positive, *n* (%)	30 (85.7)	30 (88.2)	30 (88.2)	31 (91.2)
ALT (IU/l), mean ± SD	150.1 ± 111.8	162.0 ± 127.1	142.4 ± 82.2	185.0 ± 130.8
AST (IU/l), mean ± SD	83.2 ± 40.0	114.3 ± 109.4	81.0 ± 43.0	121.6 ± 85.4
Total bilirubin (mg/dl), mean ± SD	0.65 ± 0.25	0.56 ± 0.15	0.66 ± 0.25	0.71 ± 0.28
HBV genotype (%)				
C	32 (91.4)	30 (88.2)	32 (94.1)	30 (88.2)
A	1 (2.86)	2 (5.88)	1 (2.94)	2 (5.88)
B	1 (2.86)	1 (2.94)	1 (2.94)	2 (5.88)
F	1 (2.86)	1 (2.94)	0	0

ETV entecavir; *LVD* lamivudine

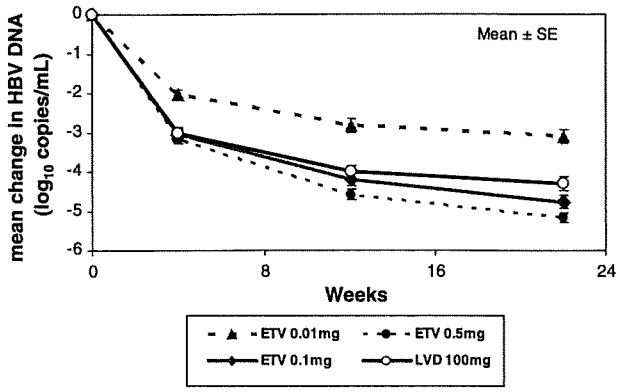

Fig. 1 Mean change from baseline in serum HBV DNA level by PCR assay through 22 weeks in patients treated with entecavir (ETV) 0.01, 0.1, and 0.5 mg and lamivudine 100 mg. Mean change in serum HBV DNA level was plotted as a function of time after the initiation of the protocol therapy (weeks). Data expressed as mean ± SE

significant dose–response relationship between log_{10} entecavir dose and reduction in log_{10} serum HBV DNA level ($P < 0.0001$).

Mean change (from baseline) in serum HBV DNA level at week 22 for the lamivudine 100 mg group was −4.29 log_{10} copies/ml (Fig. 1; Table 2). Estimated mean differences (95% CI) in serum HBV DNA level (after adjustment for baseline level and HBeAg status) were −0.39 (−0.83 to 0.05) log_{10} copies/ml between the entecavir 0.1 mg and lamivudine 100 mg groups and −0.62 (−1.06 to −0.18) log_{10} copies/ml between the entecavir 0.5 mg and lamivudine 100 mg groups, indicating the noninferiority of the entecavir 0.1 and 0.5 mg groups to the lamivudine 100 mg group and the superiority of the entecavir 0.5 mg group to the lamivudine 100 mg group ($P = 0.007$) (Table 2). In contrast, the entecavir 0.01 mg group was significantly inferior to the lamivudine 100 mg group (estimated mean difference = 1.20 [0.69–1.71]; $P < 0.0001$) (Table 2).

The secondary efficacy end point of a reduction in serum HBV DNA level 2 log_{10} copies/ml or more or HBV DNA level less than 400 copies/ml by PCR assay was achieved by 88.6% of patients in the entecavir 0.01 mg group and by 100% of patients in the entecavir 0.1 and 0.5 mg groups at week 22. Ninety-seven percent of patients in the lamivudine 100 mg group achieved this end point at week 22. HBV DNA level less than 0.7 MEq/ml by bDNA assay was achieved by 65.7%, 94.1%, and 100% of patients in the 0.01, 0.1, and 0.5 mg entecavir groups, respectively, and by 93.9% of patients in the lamivudine 100 mg treatment group.

Serologic response

Among HBeAg-positive patients, there was no significant difference between seroconversion rates at week 22 for the entecavir 0.01, 0.1, and 0.5 mg treatment groups (10.0%, 13.3%, and 3.6%, respectively) versus the lamivudine 100 mg treatment group (3.3%; Table 2). All patients who lost HBeAg also experienced HBeAg seroconversion.

Biochemical response

At baseline, elevated serum ALT levels (>1.25 × ULN) were present in more than 90% of patients in all four treatment groups. At week 22, normal serum ALT levels (World Health Organization grade 0, <1.25 × ULN) were recorded in similar proportions of patients in the entecavir 0.01, 0.1, and 0.5 mg treatment groups (75.0%, 85.3%, and 80.0% of patients, respectively) and the lamivudine treatment group (78.1% of patients), with no significant intergroup difference (Table 2).

Response

Response (HBV DNA level <0.7 MEq/ml by bDNA assay, HBeAg loss, and serum ALT level <1.25 × ULN for HBeAg-positive patients and HBV DNA level <0.7 MEq/ml by bDNA assay and serum ALT <1.25 × ULN for HBeAg-negative patients) was achieved by 14.3%, 20.6%, and 15.6% of patients in the entecavir 0.01, 0.1, and 0.5 mg

Table 2 Differences in HBV DNA levels between entecavir dose groups by PCR at week 22 in evaluable subjects

	0.1 mg ETV–0.01 mg ETV ($n = 34$, $n = 35$)	0.5 mg ETV–0.01 mg ETV ($n = 32$, $n = 35$)	0.5 mg ETV–0.1 mg ETV ($n = 32$, $n = 34$)
Estimated difference[a] (log_{10} copies/ml)	−1.61	−1.95	−0.23
Standard error	0.24	0.24	0.19
95% Confidence interval[b]	−2.20, −1.02	−2.53, −1.37	−0.69, 0.23
P-value	<0.0001	<0.0001	0.227

[a] Estimated differences are regression-adjusted for baseline serum HBV DNA and HBeAg status

[b] 95% Confidence interval is adjusted by modified Bonferroni procedures

ETV entecavir

Table 3 Virology and biochemical responses at week 22 and comparison of entecavir treatment groups with lamivudine in evaluable subjects

Response	ETV 0.01 mg ($n = 35$)	ETV 0.1 mg ($n = 34$)	ETV 0.5 mg ($n = 32$)	LVD 100 mg ($n = 33$)
HBV DNA by PCR assay				
Reduction from baseline at week 22 (log_{10} copies/ml), mean ± S.E.	−3.11 ± 0.18	−4.77 ± 0.17	−5.16 ± 0.13	−4.29 ± 0.18
HBV DNA estimated difference[a] (vs. LVD) (log_{10} copies/ml)	1.20	−0.39	−0.62	–
Standard error	0.26	0.22	0.22	–
95% Confidence interval	0.69, 1.71	−0.83, 0.05	−1.06, -0.18	–
P-value	<0.0001[b]	0.081	0.007[c]	–
HBV DNA by Roche Amplicor™ PCR assay				
Change in log_{10} HBV DNA reduction >2 or HBV DNA <400 copies/ml at week 22, *n* (%)	31 (88.6)	34 (100)	32 (100)	32 (97.0)
P-value (vs. LVD)	0.206	NR[d]	NR[d]	–
HBV DNA by Quantiplex assay				
HBV DNA <0.7 MEq/ml (2.5 pg/ml) at week 22, *n* (%)	23 (65.7)	32 (94.1)	32 (100)	31 (93.9)
P-value (vs. LVD)	0.002	1.000	NR[d]	–
Normalization of ALT levels[e]				
At week 22, *n/n* with abnormal baseline (%)	24/32 (75.0)	29/34 (85.3)	24/30 (80.0)	25/32 (78.1)
P-value (vs. LVD)	0.842	0.439	0.880	–
Loss of HBeAg and seroconversion at week 48[f]				
HBeAg loss, n/n HBeAg positive at baseline (%)	3/30 (10.0)	4/30 (13.3)	1/28 (3.6)	1/30 (3.3)
HBeAg seroconversion	3/30 (10.0)	4/30 (13.3)	1/28 (3.6)	1/30 (3.3)
P-value (vs. LVD)	0.605	0.350	1.000	–
Response[g] at week 22, *n* (%)	5 (14.3)	7 (20.6)	5 (15.6)	3 (9.1)
P-value (vs. LVD)	0.735	0.190	0.480	–

[a] Estimated differences are regression-adjusted for baseline HBV DNA and HBeAg status

[b] Two-sided test indicates inferiority of the entecavir 0.01 mg dose

[c] Two-sided test indicates superiority of the entecavir dose

[d] Not reported because expected counts <5

[e] WHO grade 0, ALT <1.25 × upper limit of normal

[f] Seroconversion was defined as disappearance of HBe-antigen and appearance of HBe-antibody

[g] Response was defined as HBV DNA levels <0.7 MEq/ml, HBeAg negativity and ALT <1.25 × ULN for HBeAg-positive patients and HBV DNA levels <0.7 MEq/ml and ALT <1.25 × ULN for HBeAg-negative patients

ETV entecavir

LVD lamivudine

treatment groups, respectively, and by 9.1% of patients in the lamivudine treatment group at week 22, and there were no significant differences in the rates of response between the four treatment groups (Table 2).

Resistance analysis

During the treatment period, serum HBV DNA level increased by 1 log_{10} copies/ml or more from its nadir in one patient in the entecavir 0.01 mg group and one patient in the lamivudine 100 mg group. Nucleotide sequence analysis of the DNA polymerase coding region, using viral samples collected from these two patients at day 1 and at week 22, revealed no lamivudine-resistance substitutions (rt180 and rt204 amino acid residues) [17, 18] or entecavir-resistance substitutions (rt184, rt202, and rt250 amino acid residues) [19].

Safety

During the study, adverse events were experienced by similar proportions of patients in the entecavir 0.01, 0.1, and 0.5 mg groups and the lamivudine 100 mg treatment group (97.1%, 97.1%, 91.2%, and 100.0%, respectively). Most adverse events were of mild or moderate intensity (grade 1/2) and transient. The most frequently reported adverse events (affecting ≥ 10% of patients in any one treatment group) included nasopharyngitis, headache, and

Table 4 Summary of adverse events and laboratory abnormalities during the 24-week blinded treatment phase

	ETV 0.01 mg ($n = 35$)	ETV 0.1 mg ($n = 34$)	ETV 0.5 mg ($n = 34$)	LVD 100 mg ($n = 34$)
Any adverse events	34 (97)	33 (97)	31 (91)	34 (100)
Most frequent clinical adverse events,[a] *n* (%)				
Nasopharyngitis	9 (25.7)	10 (29.4)	11 (32.4)	10 (29.4)
Headache	6 (17.1)	7 (20.6)	2 (5.9)	7 (20.6)
Diarrhea	1 (2.9)	1 (2.9)	4 (11.8)	4 (11.8)
Grade 3/4 clinical adverse events, *n* (%)	0	0	1 (2.9)	1 (2.9)
Grade 3/4 laboratory adverse events, *n* (%)	2 (5.7)	4 (11.8)	2 (5.9)	4 (11.8)
Any serious adverse events, *n* (%)	0	1 (2.9)	2 (5.9)	1 (2.9)
Discontinuations due to adverse events,[b] *n* (%)	0	0	1 (2.9)	1 (2.9)
ALT flares,[c] *n* (%)	0	1 (2.9)	1 (2.9)	2 (5.9)
Death, *n* (%)	0	0	0	0

[a] Occurring in at least 10% of patients

[b] One patient treated with ETV 0.5 mg discontinued the study drug due to hepatic cirrhosis. One patient treated with lamivudine discontinued due to increased ALT

[c] ALT flare defined ALT >2 × baseline and 10 × ULN

ETV entecavir

LVD lamivudine

diarrhea (Table 4). Grade 3/4 clinical adverse events occurred in one patient in the entecavir 0.5 mg group (colon carcinoma) and one patient in the lamivudine group (anal ulcer); neither of these events was considered to be related to the study drug. Serious adverse events were limited to the above-mentioned case of colon carcinoma, serum ALT elevation (entecavir 0.1 mg group [$n = 1$], entecavir 0.5 mg group [$n = 1$]), and serum aspartate aminotransferase (AST)/ALT elevation (lamivudine 100 mg group [$n = 1$]), but these were not considered to be causally related to the study drug and did not necessitate treatment discontinuation. Transient ALT flares (serum ALT >2 × baseline level and >10 × ULN) occurred in four patients (entecavir 0.1 mg group [$n = 1$], entecavir 0.5 mg group [$n = 1$], and lamivudine 100 mg group [$n = 2$]) and were associated with HBV DNA level decreases of 2 $\log_{10}$ copies/ml or more. None of the ALT flares were associated with hepatic decompensation and serum ALT and AST levels recovered to less than 1.25 × baseline level on continuation of the study treatment.

Discussion

The global ETV-005 study reported that entecavir was superior to lamivudine at reducing viral load in nucleoside-naive patients with CHB infection [15]. We conducted the present study, using an identical design to the ETV-005 study, to determine whether the findings from this earlier study are applicable to Japanese patients. In keeping with the previous findings, our results indicate that entecavir produces a dose-related reduction in serum HBV DNA level ($0.01 < 0.1 \leq 0.5$ mg) in nucleoside-naive Japanese patients with CHB; the log dose–response curves for the reduction in serum HBV DNA level with entecavir in the two studies were similar, with estimated regression curve slopes of –1.24 (Japanese study) and –1.32 (global study). In addition, both studies demonstrated the noninferiority of the entecavir 0.1 mg group compared with the lamivudine 100 mg group and the superiority of the entecavir 0.5 mg group compared with the lamivudine 100 mg group. The demonstration of a dose–response relationship for entecavir and the superiority of the entecavir 0.5 mg dose over lamivudine confirm that the antiviral activity of entecavir in Japanese patients is similar to that observed in study ETV-005. In a previous study, Ono et al. [14] demonstrated that the in vitro potency of entecavir was up to 2,200 times greater than that of lamivudine. The results presented here substantiate these earlier in vitro data and confirm the greater potency of entecavir over lamivudine in patients with CHB.

Serum ALT normalization rates with entecavir 0.5 mg and lamivudine 100 mg (~80%) were higher in the present study than those reported in the ETV-005 study (entecavir 0.5 mg, 69.0%; lamivudine 100 mg, 59.1%) [15]. In keeping with previous findings [20, 21], the incidence of entecavir-associated serum ALT flares in Japanese patients was low. The serum ALT flares occurred against a background of 2 $\log_{10}$ copies/ml or more reductions in serum

HBV DNA level, and serum ALT levels subsequently normalized without discontinuation of entecavir. Therefore, the serum ALT flare noted here may indicate recovery of the host's immune response arising from the reduction in HBV viral titer [22, 23]. ALT flares have been reported after the discontinuation of entecavir therapy [15, 16], thus necessitating long-term follow-up to identify possible posttreatment viral rebound.

In conclusion, the results of this dose-ranging study demonstrate a clear dose–response relationship for entecavir in terms of mean HBV DNA level reduction at week 22. Entecavir 0.5 mg was significantly more effective than lamivudine 100 mg in reducing HBV DNA levels in nucleoside-naive Japanese adult patients with CHB. At this dose level, entecavir treatment resulted in serum HBV DNA levels of less than 400 copies/ml in 100% of patients and normalization of serum ALT levels in 80% of patients after 22 weeks. Moreover, entecavir 0.5 mg once daily was well tolerated and showed a comparable safety profile to lamivudine.

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BMS9RJ3389

Reprinted in Japan by
Springer Healthcare Communications

Journal of Clinical Virology 47 (2010) 76–78

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Case report

Sustained virological response in a patient with chronic hepatitis C treated by monotherapy with the NS3-4A protease inhibitor telaprevir

Fumitaka Suzuki[a,*], Yoshiyuki Suzuki[a], Norio Akuta[a], Hitomi Sezaki[a], Hiromi Yatsuji[a], Yasuji Arase[a], Miharu Hirakawa[a], Yusuke Kawamura[a], Tetsuya Hosaka[a], Masahiro Kobayashi[a], Satoshi Saito[a], Kenji Ikeda[a], Mariko Kobayashi[b], Sachiyo Watahiki[b], Rie Mineta[b], Satomi Iwasaki[b], Hiromitsu Kumada[a]

[a] *Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan*
[b] *Research Institute for Hepatology, Toranomon Branch Hospital, Kawasaki, Japan*

ARTICLE INFO

Article history:
Received 22 April 2009
Received in revised form 10 July 2009
Accepted 25 September 2009

Keywords:
Hepatitis C virus
Protease inhibitor
Telaprevir
Sustained virological response

ABSTRACT

Here, we describe for the first time a case of sustained virological response (SVR) achieved in a patient with chronic hepatitis C (CH-C) by monotherapy with a NS3-4A protease inhibitor, telaprevir, without interferon therapy. A 59-year-old treatment-naïve Japanese man was enrolled in a phase II trial of telaprevir by repeat oral administration at a dose of 750 mg every 8 h for 24 weeks. At the start of treatment, he exhibited a low-level viremia with genotype 1b of the hepatitis C virus (HCV). After the first week of treatment with telaprevir, serum HCV RNA was undetectable, and negativity remained until the end of treatment. Moreover, he was evaluated as having a SVR after the post-treatment 24-week follow-up program. Two characteristics may explain the strong antiviral activity of telaprevir in the present case. First, although pre-treatment PCR-direct sequencing and cloning for the N-terminal in the NS3 region showed a protease inhibitor-resistant variant (T54A) in 1 of 32 independent clones, the T54A substitution has only a low-level resistance to protease inhibitors and his viral load was low. Second, when compared to a consequence sequence of 35 treatment-naïve patients with HCV genotype 1b, R130K and Q195K substitutions were unique to the present case. Although it is presently unknown whether the R130K and Q195K substitutions are related to SVR, this case suggests that long-term telaprevir monotherapy may be effective in CH-C patients with genotype 1 and a low viral load.

1. Introduction

The goals of antiviral treatment in patients with chronic hepatitis C (CH-C) are long-lasting eradication of the virus and a decrease in disease-related hepatic mortality. Standard treatment uses a combination of pegylated interferon and ribavirin (PEG-IFN-RBV), which provides a sustained virological response (SVR) rate of over 50%.[1,2] In Japan, approximately 70% of patients with CH-C are infected with genotype 1b, and those with a high titer of genotype 1b (≥100 KIU/mL [Amplicor; Roche Diagnostics K.K. Tokyo, Japan]) have lower rates of SVR (<50%), even on 48 weeks of PEG-IFN-RBV combination therapy.[3] Further, although treatment for CH-C is currently based on interferon (IFN), use of this agent is associated with serious adverse effects in some patients, such as mental disorders, apathy, and laboratory abnormalities.[1,2,4] Moreover, most CH-C patients in Japan over 70 years of age cannot receive IFN therapy due to either or both co-morbidities and the risk of adverse effects. For these reasons, a new treatment strategy is needed for patients with CH-C that displays high SVR rates and a favorable side-effect profile.

One recently introduced treatment strategy for CH-C is inhibition of the NS3-4A protease in the HCV polyprotein. Potential inhibitors include telaprevir (VX-950; MP-424; Mitsubishi Tanabe Pharma Co., Osaka, Japan), which has been selected as a clinical therapy candidate for the treatment of CH-C.[5] In some patients with genotype 1 and a high viral load, however, the efficacy of telaprevir monotherapy was limited, and combination therapy of telaprevir plus PEG-IFN-RBV is now standard.[6–10] On this background, we therefore report here for the first time a patient with CH-C who achieved a SVR following monotherapy with telaprevir.

2. Case report

A 59-year-old Japanese man was admitted to Toranomon Hospital, Tokyo in July 2007 following a positive result for HCV RNA at general check-up. Laboratory tests before treatment showed mild

* Corresponding author. Tel.: +81 44 877 5111; fax: +81 44 860 1623.
E-mail address: fumitakas@toranomon.gr.jp (F. Suzuki).

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doi:10.1016/j.jcv.2009.09.029

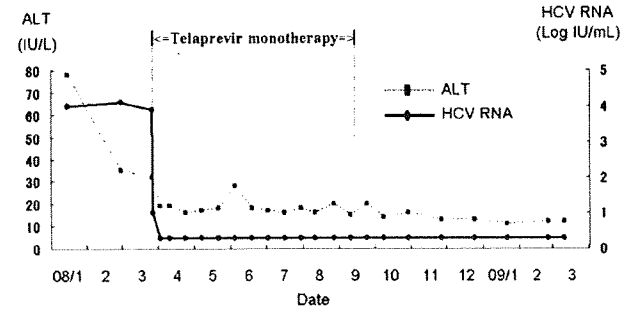

Fig. 1. Clinical course during and after 24 weeks of telaprevir monotherapy.

elevation of ALT (46 IU/L), and persistent HCV infection with genotype 1b and low-level viremia (<5 Log IU/mL [COBAS TaqMan HCV test, Roche Diagnostics K.K. Tokyo]) that continued to remain low until the start of treatment. He was diagnosed with CH-C by peritoneoscopy and liver biopsy (mild hepatitis [A1] and moderate fibrosis [F2]) at our hospital in February 2008. He had not received IFN therapy or any other antiviral drugs, and was enrolled in a phase II trial of telaprevir. Written informed consent was obtained, and the study was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki. Treatment with telaprevir was started in March 2008, at which time serum HCV RNA was 3.9 Log IU/mL. Treatment was by repeat oral administration at a dose of 750 mg every 8 h for 24 weeks. Serum HCV RNA was undetectable after the first week and remained negative until the end of treatment (September 2008), and moreover remains undetectable as of March 2009. He was evaluated as having a SVR after the post-treatment 24-week follow-up program (Fig. 1).

The genome sequence for the N-terminal 609 nucleotides (203 amino acids) in the NS3 region of HCV isolates from the patient was analyzed before treatment with telaprevir. HCV RNA was extracted from 100 μL of serum and the nucleotide sequences were determined by direct sequencing and cloning. The primers used to amplify the NS3 region were NS3-F1 (5′-ACACCGCGGCGTGTGGGGACAT-3′; nucleotides 3295–3316) and NS3-AS2 (5′-GCTCTTGCCGCTGCCAGTGGGA-3′; nucleotides 4040–4019) as the first (outer) primer pair and NS3-F3 (5′-CAGGGGTGGCGGCTCCTT -3′; nucleotides 3390–3407) and NS3-AS2 as the second (inner) primer pair.[11] Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 s at 95 C, annealing of primers for 1 min at 63 C, extension for 1 min at 72 C, and final extension was performed at 72 C for 7 min. PCR-amplified DNA was purified after agarose gel electrophoresis and amplification products of the second-round PCR were ligated with plasmid and transformed in *Escherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). Dideoxynucleotide termination sequencing was performed with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems Japan, Tokyo). Sequences of 32 independent clones from the sample were determined and analyzed. The pre-treatment analyses by PCR-cloning showed a variant (T54A) resistant to protease inhibitors in 1 of the 32 clones.

We also made a consensus sequence of the NS3 region from the PCR-direct sequences of 35 treatment-naïve Japanese patients with HCV genotype 1b in our hospital (Fig. 2). Compared to the consensus sequence, there were a total of 5 identical substitution variants (V48I, P89S, S122G, R130K, Q195K) within the 32 independent clones from this patient, among which R130K and Q195K were unique to this patient.

3. Discussion

Previous studies showed that telaprevir monotherapy for HCV patients with genotype 1 and a high viral load demonstrated substantial antiviral activity, and the median maximum change was −4.77 Log IU/mL with administration at 750 mg every 8 h for 2 weeks.[6,7] In Reesink et al., HCV RNA decreased below the limit of

```
                1       10        20        30        40        50
CONSENSUS       APITAYSQQT RGLLGCIITS LTGRDKNQVE GEVQVVSTAT QSFLATCVNG
Case clone1     ---------- ---------- ---------- ---------- -------I--
Case clone2     ---------- ---------- ---------- ---------- -------I--
Case clone3     -----H---- ---------- ---------- ---------- -------I--
Case clone4     ---------- ---------- ---------- ---------- -------I--
Case clone5     ---------- ---------- ---------- ---------- -------I--
                51                                                100
CONSENSUS       VCWTVYHGAG SKTLAGPKGP ITQMYTNVDQ DLVGWQAPPG ARSLTPCTCG
Case clone1     ---------- ---------- ---------- --------S- ----------
Case clone2     -----F---- ---------- ---------- --------S- ----------
Case clone3     ---A------ ---------- ---------- --------S- ----------
Case clone4     ---------- ---------- ---------- --------S- -----L----
Case clone5     -----F---- ---------- ---------- --------S- ----------
                101                       130               150
CONSENSUS       SSDLYLVTRH ADVIPVRRRG DSRGSLLSPR PVSYLKGSSG GPLLCPSGHA
Case clone1     ---------- ---------- -G-------K ---------- ----------
Case clone2     ---------- ---------- -G-------K ---------- ----------
Case clone3     ---------- ---------- -G-------K ---------- ----------
Case clone4     ---------- ---------- -G-------K ---------- ----------
Case clone5     ---------- ---------- -G-------K ---------- ----------
                151                                   195  200
CONSENSUS       VGIFRAAVCT RGVAKAVDFI PVESMETTMR SPVFTDNSSP PAVPQTFQVA
Case clone1     ---------- ---------- ---------- ---------- ----K-----  15
Case clone2     ---------- ---------- ---------- ---------- ----K-----  14
Case clone3     ---------- ---------- ---------- ---------- ----K-----   1
Case clone4     ---------- ---------- ---------- ---------- ----K-----   1
Case clone5     ---------- ---------- ---------- ---------- ----K----V   1
```

Fig. 2. Evolution of the HCV NS3 gene sequence at the start of telaprevir monotherapy. Consensus sequence was made from the HCV RNA of 35 treatment-naïve Japanese patients with genotype 1b in our hospital. The number of clones within each sample of identical amino acid sequences is given on the right at the end of each sequence. Dashes indicate identical amino acid sequences.

detection (10 IU/mL) for 2 patients in the group receiving 750 mg every 8 h.[6] In some patients, however, HCV RNA levels increased between days 7 and 14, and mutations that confer resistance to telaprevir were detected. This trial of telaprevir monotherapy was therefore terminated after 2 weeks, and combination therapy of telaprevir plus PEG-IFN-RBV is now used in the USA and Europe.[8–10] Our case may therefore represent an unusual and possibly serendipitous response to long-term telaprevir monotherapy, and the efficacy of monotherapy remains unclear.

To our knowledge, this is the first report of a patient with CH-C achieving SVR by telaprevir monotherapy, without the use of IFN. Three treatment-naïve Japanese patients were enrolled in our hospital for this phase II trial of telaprevir monotherapy over 24 weeks. Before treatment, the 2 non-SVR patients had a high HCV RNA viral load (>5 Log IU/mL), while the viral load in the SVR patient remained low. Further, while HCV RNA decreased below the limit of detection (10 IU/mL) and negativity of HCV RNA remained until the end of treatment in 2 patients, HCV RNA in the other non-SVR patient reappeared after treatment cessation.

The development of drug resistance has been a challenge for treatment strategies in many viral infections. The high replication rate and the error-prone nature of viral RNA polymerases generate a viral quasi-species from which variants resistant to viral inhibitors can be selected. Recently, Kuntzen et al. reported that viral loads were high in the majority of treatment-naïve patients carrying mutations of protease and polymerase inhibitors.[12] Low viral load may therefore be an important factor for achieving SVR by telaprevir monotherapy.

It has recently been reported that CH-C patients never treated with an NS3-4A protease inhibitor may nevertheless possess variants resistant to protease inhibitors involving the HCV RNA NS3 region.[12–14] While there was a resistant variant (T54A) in this case, this mutation exhibits only low-level resistance,[7] and the number of mutant variants may have been few along with substantial suppression of HCV replication by telaprevir. This may also help to explain the effectiveness of telaprevir in this case.

Moreover, two amino acid substitutions (R130K and Q195K) were unique to this patient. We therefore checked the nucleotide sequence data in the DDBJ/EMBL/GenBank databases and found a previous report by Franco et al. on the R130K substitution (EF013801, EF013863, EF013867, EF013869).[15] Interestingly, although only a minor clone (4% of total) in that study, the viral load of the patient with the R130K substitution was also low (2364 IU/mL). To date, however, the Q195K substitution has not been reported. Their presence in this case may indicate that telaprevir has a stronger antiviral activity against HCV with these substitutions.

The NS3-4A protease targeted by protease inhibitors is required for viral polyprotein processing, an essential step in viral replication, but is also responsible for disrupting IFN responses to the infection.[16] Previous studies have shown that high concentrations of protease inhibitors may restore retinoic acid-inducible gene I (RIG-I) signaling in HCV replicon cells, [16–18] and Liang et al. also recently reported that protease inhibitors could restore interferon regulatory factor 3 (IRF-3) signaling in HCV-infected cells.[19] In our patient, telaprevir may have therefore rescued the NS3-4A-mediated blockade of IRF-3 signaling *in vivo*.

Further studies are required, such as sequencing analyses of the HCV NS3 region, and research into the rescue of IFN-β signaling through the RIG-I pathway. It is foreseeable in the future for CH-C patients to be treated by one or a combination of two or more oral drugs with high efficacy and genetic barriers to resistance and low side-effect profiles. Telaprevir may hold promise for being one of these drugs, even if only within a subset of patients, and further studies into telaprevir monotherapy or combination therapy with other oral drugs is therefore warranted. Although still an isolated response, based on our current molecular understanding of HCV infection and pharmacotherapy, this case suggests that long-term telaprevir monotherapy may be effective in other CH-C patients with genotype 1 and a low viral load.

Conflict of interest

The authors have no commercial or other associations that may pose a conflict of interest.

Acknowledgments

This study was supported in part by a grant-in-aid from the Ministry of Health, Labor and Welfare, Japan.

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